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# Supplemental Data

# The Prototoxin *lynx1* Acts on Nicotinic Acetylcholine Receptors to Balance Neuronal Activity and Survival In Vivo

**Julie M. Miwa, Tanya R. Stevens, Sarah L. King, Barbara J. Caldarone, Ines Ibanez-Tallon, Cheng Xiao, Reiko Maki Fitzsimonds, Constantine Pavlides, Henry A. Lester, Marina R. Picciotto, and Nathaniel Heintz**

## Supplemental Materials and Methods:

### Targeted mutagenesis:

A 9kb HindIII/lynx1fragment was shotgun subcloned from lynx1 BAC26-1 (Miwa et al., 1999) into pBluescriptKS$^{+}$ vector. Gene specific primers, GCTGCTGACC TCCTATTCAC TCTGGCACTG CCCTCACGTC ACGCGTTCTG CAAACCCTAT GCTACTCCGTCG and GCTGGGACCA GGGCCAAGGT CACCGGGGTA GCAAAGCCAG CAATATTCAT ATGTCCCGGC GGATTTGTCC TACTCAGGAG AGCG were used to PCR amplify from frt-neo-recA plasmid and the resulting PCR product was electroporated into 322EC cells, containing the 9kb lynx1 HindIII pKS$^{+}$ construct. Modification was carried out using a bacterial recombination method (Yu et al., 2000). The targeting construct was electroporated into 129svj embryonic stem (ES) cells, which were injected into blastocysts and implanted into pseudopregnant females. Chimeras were crossed to C57Bl6/J mice to produce F1(129svj/C57Bl6/J) *lynx1* $^{+/-}$ mice. Introduction of the neo gene created an additional Ssp1(S) restriction site which was exploited for use in screening for recombinants. 3' external probe was generated by PCR amplification from genomic DNA using primers CAATATGGATGCCTTCCCTACT and CAAGGAGGTAAGGAGCGTGTC primers.

Western analysis utilized purified anti-lynx pAb (1:1000) (Ibanez-Tallon et al., 2002, 2004) to probe 10 μg of total homogenate from *lynx1*$^{-/-}$ and *lynx1*$^{+/+}$ adult forebrain extracts, and detected with goat anti-rabbit HRP conjugated secondary antibodies

(Jackson labs).*In situ* hybridization with coding probe showed no lynx1 message in *lynx1*$^{-/-}$ brains sections (data not shown).

**Whole-cell electrophysiological recordings:**

*lynx1*$^{+/-}$ mice crossed 11 generations to C57Bl6/J (C57Bl6/J$^{11}$ *lynx1*$^{+/-}$ mice were crossed to one another to produce C57Bl6/J *lynx1* $^{-/-}$ mice. C57bl6/J mice were used as controls for these experiments. Somatic whole-cell recordings were made from visually identified habenular neurons from P14-17 mice. Internal pipette solution, in mM: 100 mM $K^+$ Gluconate, 0.1 $CaCl^{2+}$, 1.1 EGTA, 5 $MgCl^{2+}$, 10 HEPES, 3 ATP, 3 phosphocreatine, 0.3 GTP, pH to 7.2 with KOH. The resistance of the electrodes was 5 - 8 MΩ. Series resistance was compensated 70-85% using lag values of 7-8 μs. Nicotine was applied using a glass pipette connected to a relay-controlled pressure device (Picospritzer, Parker Hannafin, Cleveland OH 44124-4141); this pipette was moved to within 20 μm of the recorded cell using a piezoelectric manipulator (Burleigh, Toronto CA) over a period of 250 ms starting 300 ms prior to drug application (Tapper et al., 2004). Nicotine was then applied for 250 ms at 10-30 psi. 50 ms after the end of this puff, the glass pipette was retracted over a period of 250 ms. This procedure minimized desensitization due to leak from the puffer pipette between pulses. Mecamylamine was bath perfused at a concentration of 10 μm in ACSF for 3 min.

**$Ca^{2+}$ Imaging:**

Mixed cortical cultures were made from wt or *lynx1*$^{-/-}$ fetal mice (E16-18) as has been described (Stevens et al., 2003). See supplemental material. Neurons were dissociated by incubating minced cortices in PDD for 15 min at 37$^{o}$C, and incubated for 11-13 days. Glial cells were present in the cultures but were less then 10% of the overall population.

Following nicotine or HBS pre-treatment, cells were loaded with 2μM fluo-3 (Molecular Probes, Eugene, OR) for 10 min at RT in HBS 0/0 and washed twice with HBS 2/1 (HBS with 2 mM $CaCl_2$ and 1 mM $MgCl_2$), 37 $^{o}$C, flow rate 2 ml./min All images were obtained using a 20x objective on a Nikon TE300 inverted microscope equipped with a cooled CCD camera (Hamamatsu orca), collected at 2 sec intervals and adjusted for the

level of background intensity, digitally colored and analyzed using IPLab software (Scanalytics, Fairfax, VA).

To assess the effect of chronic nicotine treatment, cells were treated with 10μM nicotine or buffer for 1 hr and subsequently loaded with 2μM fluo-3 (Molecular Probes/ www.probes.com) for 10 min at room temperature (RT). IPLab software measured somatic pixel density, adjusted against background, for over 40 neurons per genotype/condition.

For acute nicotine experiments, $Ca^{2+}$ influx was determined as the change from basal levels upon a 10 sec application of 1mM nicotine ([stimulated level – basal level] /basal level of fluorescence). Change in fluorescence was quantitated as described by Vijayaraghavan et al.(1992). Stacked images from 0-60 sec were collected for each experiment. A region including the entire soma of an individual neuron was selected and the change in pixel density was averaged over this area for every frame. *lynx1*$^{-/-}$ n=18, wt n=24.

Similar to the acute nicotine experiments, a dose-response measurement was determined for *lynx1*$^{-/-}$ cultures. In these experiments the extracellular $Ca^{2+}$ was increased to a final concentration of 3mM and 0mM $MgCl^{2+}$, to increase the changes observed in $Ca^{2+}$ influx at lower doses. In these studies nicotine (1mM-1x$10^{-6}$mM) was applied for 10 sec and the change in fluorescence was determined for each concentration. The wt cultures did not show a significant change in fluorescence with highest application of 1mM nicotine ($\Delta F/F=0.04$ n=24 cells), so lower doses of nicotine were not tested. These experiments were conducted in three different cultures. 1mM n=32 cells, 0.1 mM n=20 cells, 10 μM n=32 cells, 1 μM n=28 cells. To verify the presence of cholinergic neurons, immunofluoresence analyses were conducted on wt cultures, indicating the presence of all of these components (data not shown).

**Hippocampal Slice physiology:**

*lynx1*$^{+/-}$ mice crossed 8 generations to C57Bl6/J (C57Bl6/J$^{8}$ *lynx1*$^{+/-}$ mice were crossed to one another to produce C57Bl6/J *lynx1*$^{+/+}$ (wt) and *lynx1*$^{-/-}$ (lynx1 KO) mice. Transverse hippocampal slices (300 μm) were prepared on a McIllwain tissue chopper and kept submerged in ACSF (in mM 124.0 NaCl, 5.0 KCl, 2.4 CaCl2, 1.3 MgSO4, 10 NaHCo3, 1.25 NaH2P04, 10 glucose). A heated interface chamber (32 C) was used to record field potentials in CA1 stratum pyramidalis using a glass micropipettes filled with 3M NaCl (1-3 MΩ). The field potentials were amplified with an AC differential amplifier with low-pass filter set a 3 kHz and high-pass at 30 Hz and stored using the Labview program on an Apple Computer for analysis. An input-output curve was taken between minimum and maximum responses, and a test stimulus intensity was chosen at no greater than half-maximal response. Paired stimuli were applied at interspike intervals of 10 to 70 ms. PPF ratios were calculated as the amplitude of the population response to the second stimulus over the amplitude of the population response to the initial stimulus.

**Behavior:**

Heterozygous F1 (129svj/C57Bl6/J) *lynx1*$^{+/-}$ mice were bred to produce F2 (129svj/C57Bl6/J) *lynx1*$^{+/+}$ and *lynx1*$^{-/-}$ genotypes. At least 8 adult males, housed together under the same conditions as their littermates of each genotype were tested, and the experimenter was blind as to genotype. Fear conditioning : see Caldarone et al., 2000. Fear conditioning was assessed in a *Med Associates* conditioning chamber Approximately 24 hr after conditioning, mice were tested for contextual conditioning by scoring freezing behavior for 5 min in the conditioning chamber. For the cued test, mice were placed into a 29x18.5x13 cm clear plastic cage that was covered with a filter lid, and a novel odor (orange extract) was added to the context. Mice were scored for freezing in the altered context for 3 min before and 3 min during presentation of the tone (cued conditioning test). Freezing, sampled every 10 secs, was defined as the absence of all movement except for respiration for a minimum of 1 sec.

**Passive Avoidance learning:**

Passive avoidance training was performed as has been described (King et al., 2003). Testing was carried out in a two-chambered (one light and one dark chamber) mouse PA

chamber (Ugo Basile, Comerio, Italy). On the training day, the mouse was placed in the light chamber and latency to enter the dark chamber was measured, whereupon the door between compartments closed and a 2 sec electric shock (0.2mA) was administered through the grid floor. On the test day, mice were again placed in the light chamber and time to enter the dark chamber was recorded. Morris Water Maze: see Rabenstein et al., 2005. Path length, time spent in each quadrant, and latency to find the platform were measured by a Poly-track Video Tracking System (San Diego Instruments, San Diego, CA). Animals that did not find the platform within 60 sec were placed on the platform manually, and trained for 4 trials per day. After training, the platform was removed and mice were placed in the middle of the tank and allowed to swim for 60 sec (transfer test). Time spent in each quadrant as well as number of platform crosses was recorded. Elevated Plus Maze Analyses: All mice used were naive to the apparatus. At the beginning of each trial, the mouse was placed on the central area facing an open arm and allowed to explore the maze for 5 min. All tests were recorded with a camera and analyzed later by an observer blind to the experimental group of each mouse. The following measures were recorded: number of total arm entries, number of open arm entries, number of closed arm entries, and time spent in open arms. Locomotor analyses: Diurnal locomotor activity was measured in the homecage as has been described (King et al., 2004). The locomotor apparatus consisted of 6 photocells, spaced 4 cm apart. Locomotor activity, as assessed by the number of beam breaks, was collected in 1 hr blocks. Animals were housed singly in their home cage and measured for a period of 72 hours. Results were collected and for each animal, a consecutive 24 hour period was averaged over the three 24 hour periods. For locomotor activity in response to novelty, mice were placed into a clear Plexiglas box (40x40x17 cm) and locomotion was measured for 20 min by infrared beams (Columbus Instruments, Columbus, OH), on two consecutive days. Rotarod: Animals were housed in the same room as the testing occurred. *lynx1*$^{-/-}$ and wt mice were administered either 2% saccharin, or 200 mg/ml of nicotine hemisulfate salt in 2% saccharin (Sigma, St Louis, MO), through their drinking water. Measurements were made on the volume of either saccarin or nicotine solution. There was a significant reduction in nicotine/saccharin intake as compared to saccharin intake in the initial 4 weeks, but this difference was reduced in subsequent weeks of

nicotine administration. Adult mice were tested in squads of 4, for 8 trials per day on consecutive days. Animals were placed on an initially stationary rod of an Economex rod for mice (Columbus Instruments), and tested with an accelerating paradigm with a starting speed of 1 RPM and a rate of acceleration of 0.1 RPM/s. Animals were scored for time to fall, or time to stop running. If the animals stopped running and gripped the bar, the time was stopped after one revolution. In order to control for the possibility that differences in nicotine administration between wt and *lynx1*$^{-/-}$ mice, one squad of animals were segregated by genotype during the nicotine administration phase. In these studies, there were no significant differences in nicotine administration between wt and *lynx1*$^{-/-}$ mice.

**Histology:**

For the aging experiment, progeny from N1 (129svj/C57Bl6/J) *lynx1*$^{+/-}$ x N1 (129svj/C57Bl6/J) *lynx1*$^{+/-}$ were used, including but not limited to the animals which had undergone behavioral testing. Animals were perfused with 4% paraformaldehyde in PBS or cacodylate buffer trans-cardially, and post-fixed o/n. Brains were sectioned coronally and stained with H&E (NeuroscienceAssociates/ neuroscienceassociates.com). Micrographs were taken with a Zeiss Axioplan2 microscope with a 10x objective and an automated x,y,stage (Marzhauser) controlled by Zeiss KS400 software to stitch the individual pictures together.

For quantitation of the vacuolation, sections were picked at the same rostral- caudal level through the striatum. Bregman 0.00 Interaural 3.80 mm. The area of measurements occurred at the striatum bounded by the corpus callosum to the level of the posterior commissure. The number of vacuoles was counted in the left and right striatum for each section, yielding two data points per brain and was conducted blind as to genotype. Quantitation was conducted under the same bright light conditions for each section counted, at 10x magnification in the region bounded by the above landmarks were counted to yield each data point. The number of gaps in the neuropil of at least a cell body in diameter were counted, to yield the number of vacuoles per striatal hemisphere. Vacuolation in *lynx1*$^{-/-}$ mice was observed in lines bred to 129svj mice for 4 generations,

C57bl6/J background for 5 generations, and in progeny from N1(129svj/C57Bl6/J) *lynx1*$^{+/-}$ x N1(129svj/C57Bl6/J) *lynx1*$^{+/-}$ mice, as were used in the behavioral analyses. This effect was observed independent of background (129svj, C57Bl6/J, or 129svj/C57Bl6/J backgrounds), or fixation solution (paraformaldehyde in PBS or cacodylate buffer, formaldehyde, formalin), fixation method (transcardial perfusion, paraffin embedding or drop fixation). No alterations in vascularization were detected in *lynx1*$^{-/-}$ brains.

For analysis of nicotine-treated mice F2 (129svj/C57Bl6/J) *lynx1*$^{-/-}$ were housed with F2 (129svj/C57Bl6/J) *lynx1*$^{+/+}$ littermates. Drinking water contained 200 μg/ml nicotine in 2% saccharin (Sigma), starting at the age of 8 mnths for a 10 mnth period of time (see above). Nicotine consumption was monitored and continued until time of dissection, at 1.5 years old. 3-10 animals were used for this analysis.

α7 and β2 nAChR$^{-/-}$ mice were obtained from the Beaudet laboratory and bred to C57bl6 wt mice (Charles River labs). *Lynx1*$^{-/-}$ mice which had been backcrossed to C57Bl6/J for 6 generations (C57Bl6/J6 *lynx1*$^{+/-}$) were crossed to produce C57Bl6/J6 *α7* nAChR/lynx1$^{+/+\ -/-}$ or $^{-/-\ -/-}$ and β2 nAChR/lynx1$^{+/+\ -/-}$ or $^{-/-\ -/-}$ mice. 15 mth old mouse brains were drop fixed in 4% paraformaldehyde in PB. wt n=5, *β2 nAChR*$^{-/-}$*/lynx1*$^{-/-}$ n=6, *α7 nAChR*$^{-/-}$*/lynx1* $^{-/-}$ n=5, *α7 nAChR*$^{-/-}$ n=2, *β2 nAChR*$^{-/-}$ n=2.

Silver stain of degenerating neurons was conducted by a modification of DeOlmos amino cupric silver stain for disintegrative neuronal degeneration method (Neuroscience Associates, Switzer, 1993, DeOlmos and Ingram, 1971). Animals were perfused with 4% paraformaldehyde in cacodylate buffer trans-cardially, and skulls post-fixed o/n, then dissected and post-fixed again o/n. Brains were sectioned coronally, and stained every 24$^{th}$ section, stained en block, and visualized under the same bright light conditions for all sections at each level. Micrographs of the dorsal striatum, taken at 20x magnification, were recorded digitally, and the images quantitated using NIH image. Density measures of the *lynx1*$^{-/-}$ brains were normalized against the wt measures.

**Supplemental References**

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